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BIOSILICA-IMMOBILIZED ENZYMES FOR BIOCATALYSIS

Lorena Betancor
Department of Biochemistry, University of Cambridge
80 Tennis Court Road, Old Addenbrookes Site
Cambridge CB2 1GA United Kingdom

Heather R. Luckarift
Air Force Research Laboratory

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Air Force Research Laboratory
Materials and Manufacturing Directorate
Airbase Technologies Division
139 Barnes Drive, Suite 2
Tyndall AFB, FL 32403-5323

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80 Tennis Court Road, Old Addenbrookes Site
Cambridge CB2 1GA United Kingdom

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14. ABSTRACT

Bacterial enzymes are remarkable biocatalysts and catalyze a wide variety of processes that can be utilized for the production of novel compounds or pharmaceutical intermediates. Enzymes also possess a wide range of pharmacological activities and are often investigated for therapeutic effects. A stable immobilized-enzyme preparation is essential to facilitate the use of enzymes in potential applications. Biomineralization reactions have been demonstrated as an effective mechanism to generate silica nanoparticles which are suitable for enzyme immobilization. Biological templates are used to catalyze the precipitation of silica to form a network of fused silica nanospheres. Additional enzyme added during the reaction becomes rapidly entrapped inside the silica spheres as they form. The silica forms at ambient environmental conditions, providing a biocompatible environment for enzyme immobilization. The silica-enzyme immobilization technique provides significant stabilization to a wide range of enzymes. The applicability of silica-encapsulated enzymes is presented for a range of model systems to provide insight into the versatility of the method for biocatalysis.

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BIOSILICA-IMMOBILIZED ENZYMES FOR BIOCATALYSIS

Lorena Betancor [1] and Heather R Luckarift [2]

[1]*Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Old Addenbrookes Site, Cambridge CB2 1GA* [2]*Air Force Research Laboratory, 139 Barnes Drive, Suite # 2, Tyndall AFB, Florida 32403*

Correspondence should be addressed to lb379@mole.bio.cam.ac.uk and hluckarift@gtcom.net

ABSTRACT

Bacterial enzymes are remarkable biocatalysts and catalyze a wide variety of processes that can be utilized for the production of novel compounds or pharmaceutical intermediates. Enzymes also possess a wide range of pharmacological activities and are often investigated for therapeutic effects. A stable immobilized-enzyme preparation is essential to facilitate the use of enzymes in potential applications. Biomineralization reactions have been demonstrated as an effective mechanism to generate silica nanoparticles which are suitable for enzyme immobilization. Biological templates are used to catalyze the precipitation of silica to form a network of fused silica nanospheres. Additional enzyme added during the reaction becomes rapidly entrapped inside the silica spheres as they form. The silica forms at ambient environmental conditions, providing a biocompatible environment for enzyme immobilization. The silica-enzyme immobilization technique provides significant stabilization to a wide range of enzymes. The applicability of silica-encapsulated enzymes is presented for a range of model systems to provide insight into the versatility of the method for biocatalysis.

INTRODUCTION

Biocatalysis capitalizes on the metabolic diversity of natural enzymes for commercial realization of specialty products. Enzymes catalyze a remarkably versatile range of catalytic reactions, often with precise stereo- and regio-selectivity. In addition, the natural substrate specificity of many enzymes can be extended to non-natural substrates by genetic engineering. All of these factors provide a wealth of catalytic processes that can be utilized to produce fine chemicals and pharmaceuticals that are otherwise unobtainable or impractical by conventional synthesis. As such, products derived from biocatalysis now feature predominantly in our lives, in products as diverse as laundry and dishwasher detergents to stonewashing of denim and clarification of wine (Table 1) [1-3].

Table I: Examples of enzymes catalysis in common household items

Enzyme	Application
Lipases, Amylases, Proteases, Cellulases	Laundry and dishwasher detergents
Catalases	Neutralizes peroxide in contact lens solutions
Cellulases	Stonewash finish of denim
Xylanases	Pulp and paper manufacturing
Phytases	Improves nutritional value of animal feeds
Lipases, Acylase	Drug products and pharmaceutical intermediates
Amylase	Manufacture of glucose syrups and starch modification
Maltogenic alpha-amylase	Improves shelf life of bread
Lipoxygenase	Used for bleaching and strengthening of dough
Lipases, Proteases	Accelerates cheese ripening
Glucose Isomerase	Production of high fructose corn syrup
Pectinases	Fruit juice processing and clarification of wine

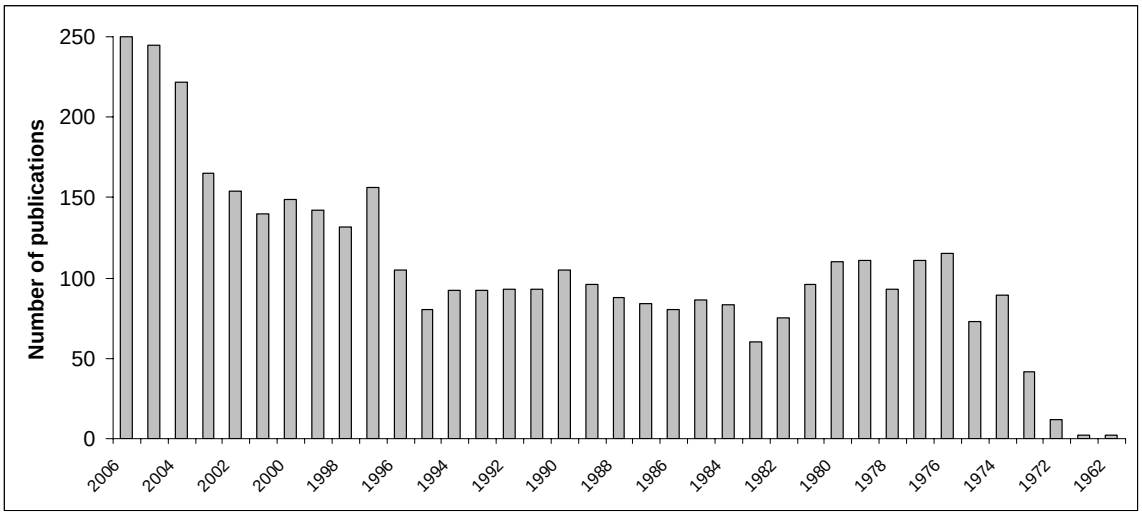
Significant advancements in genetic and protein engineering have provided a wealth of catalytic enzymes with enhanced characteristics, such as improved protein stability and defined substrate specificity to eliminate unwanted side-reactions. In many cases, however, enzymes operate at benign environmental conditions, i.e. room temperature, neutral pH and under aqueous conditions. The utilization of enzymes in biocatalysis, particularly at an industrial scale has therefore necessitated the development of effective strategies to immobilize enzymes and retain activity in non-physiological reaction conditions. In addition to stabilization, enzyme immobilization also allows for reuse of the biocatalyst, leading to a significant reduction in cost. Enzyme immobilization also extends the utility of enzyme reactions to a range of non-physiological environments, such as extreme temperatures, non-aqueous solvents, ionic fluids and supercritical CO_2 [4-10].

Enzyme immobilization

The methodology to stabilize enzymes by entrapment and encapsulation emerged almost 50 years ago and following a recommendation made at the 1973 Enzyme Engineering Conference (Henniker, New Hampshire) the term “immobilized enzymes” was readily adopted [11]. Since then, interest has escalated as an ever increasing diversity of applications, novel enzyme supports and immobilization strategies has become

available. As such, enzyme immobilization has become a field of general interest for a wide range of scientific fields including; biotechnology, biochemistry, chemistry, biology and engineering. In the last decade the number of publications related to this area has increased rapidly with indications that developments in enzyme immobilization are still far from exhausted (Figure 1).

Figure 1. Number of publications related to enzyme immobilization over the past 50 years (Source: Scifinder**)**



Enzymes are, by nature, soluble entities although specific examples such as membrane-associated proteins prefer a lipophilic environment for natural stabilization. The advantages of enzyme 'insolubilization' overcome major problems which are inherent with the use of soluble enzymes such as product contamination, difficulty of separation from a reaction mixture and as mentioned before, poor stability and limited reuse. Moreover, if properly designed, immobilization may also improve enzyme properties as substrate specificity and selectivity may be enhanced and the effect of inhibitors can be reduced. Many methods of immobilization and entrapment cause significant structural deformation of the enzyme, leading to reduction in activity. Significant optimization of the immobilization method is therefore often required and factors such as stability may be sacrificed in favor of increased loading capacity [12,13]. For further reading on the advantages of enzyme immobilization the reader is referred to several extensive recent review articles [14-17].

The major techniques for immobilization reported in the literature include covalent binding, ionic and hydrophobic adsorption, aggregation and entrapment. Although, the requirements for different enzymes may vary and specific conditions may be needed for a particular application, it is possible to generalize certain characteristics for each methodology. Multipoint covalent attachment is one of the most effective methods available for thermal stabilization of immobilized enzymes [18-21]. The structure of a multipoint immobilized enzyme molecule becomes more rigid, preventing the molecular movements that typical lead to conformational changes and enzyme inactivation [14,22,23]. There are a few reports, however, in which the use of stable covalent bonds for the attachment of an enzyme to a support proved to be detrimental to

enzyme activity and reduced the stability of the biocatalyst [24,25]. Immobilization that occurs through ionic and hydrophobic interactions provides a much less permanent fixture than covalent attachment but has the advantage of being reversible; allowing the support to be reused after the enzyme has been inactivated [26-30]. Ionic and hydrophobic interactions can also confer additional stability against denaturation in non-physiological environments [31] and provide a protected microenvironment that can enhance the catalytic properties of the biocatalysts [30,32,33]. The weak binding, however, between the enzyme and support has a disadvantage in that the absorbed enzyme may leach from its support upon a change in the reaction environment, such as pH, ionic strength or temperature.

In any approach to immobilize enzymes, the goal is to achieve a high specific activity without compromising the other advantages of immobilization (such as higher stability). In recent years, a number of methods have been reported that achieve higher volumetric activities; the most efficient being physical aggregation of enzymes and subsequent chemical cross-linking [34]. CLEAs (cross-linked enzyme aggregates) are easy to prepare and as non supported biocatalysts represent an alternative to the use of expensive supports [17]. Several enzymes have been successfully immobilized using this strategy including penicillin G acylase [35-37], lipases [38] and nitrilases [39]. The diversity of enzymes able to form active CLEAs, however, may be restricted by their ability to resist chemical cross-linking and diffusion limitations due to aggregation must be overcome [39].

Silica has been widely demonstrated as an inert and stable matrix for enzyme immobilization due to high specific surface areas and controllable pore diameters that can be tailored to the dimension of a specific enzyme; i.e. microporous (<2 nm pore size), mesoporous (2 – 50 nm pore size) or macroporous (> 50 nm pore size) silica. As most enzymes are of the order of 3 - 6 nm in diameter, mesoporous materials are most commonly used [40-44]. Silica sol-gels have proved to be a versatile alternative and sol-gel encapsulation has been demonstrated for a wide variety of biomolecules with particular relevance to the development of biosensors [40,45-48]. The sol-gel process is primarily used for making glass and ceramic composites but can be fabricated to produce powders, fibers, membranes, thin film coatings and aerogels. For further reading on sol-gel encapsulation of enzymes and their diverse applications, the reader is referred to a series of recent reviews [45,47,49-56]. One of the primary limitations of the sol-gel technique is poor loading efficiency and enzyme leakage. The problem has in some instances been addressed by designing protocols for the preparation of matrixes with a pore size adequate to allow the flow of substrates and products but small enough to prevent the elution of the entrapped biocomponent [57,58]. Using a similar sol-gel technique but based around a biological template, we have demonstrated a biological silicification reaction that provides a biocompatible and simple method for enzyme encapsulation resulting in stable catalysts with enhanced mechanical stability and high volumetric activity [59,60]. The ‘bio’-silicification reaction results in the formation of silica nanospheres (‘bio’-silica) that causes the physical entrapment of biomolecules. The method appears to limit negative interactions and allows high recoveries of enzyme activity and improved thermal stability [59,61,62]. The application of silicification as a method for enzyme immobilization will be discussed in more detail below.

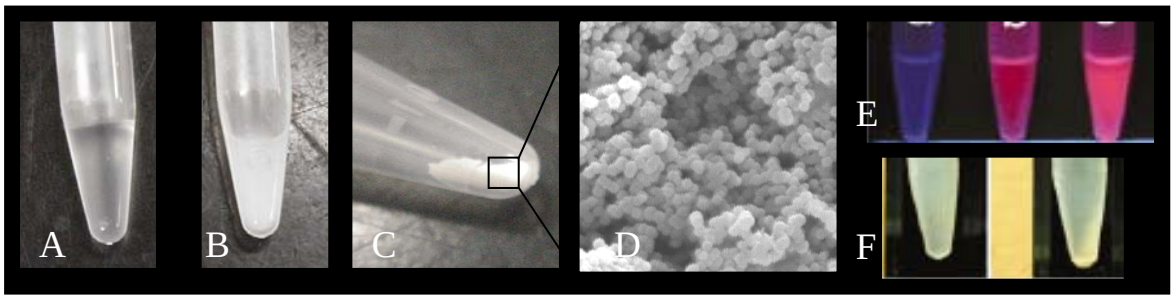
Enzyme immobilization in biosilica

Recent interest in nanotechnology has provided a wealth of interesting nano-scaffolds which could potentially support enzyme immobilization and as such, reports of enzymes immobilized to nano-sized scaffolds such as spheres, fibres, tubes and single enzyme particles have been demonstrated [40,63-65]. The premise of using nano-scale structures for immobilization is to reduce diffusion limitations and maximize the functional surface area to increase enzyme loading. In addition, the physical characteristics of nanoparticles, such as enhanced diffusion characteristics and particle motility, can impact the inherent catalytic activity of attached enzymes [66].

The work documented herein focuses primarily on recent advances in the biologically-templated formation of silica nanoparticles, which have proved to be a versatile and widely applicable new technology for enzyme immobilization. In nature, biomineralization provides a mechanism in which biological organisms generate hard composite materials (*e.g.*, shells, bones and teeth) by using proteins as scaffolds for inorganic materials. When silica is the product; the reaction is termed biosilicification; a process that has been extensively studied, particularly in diatoms and marine sponges [67-72]. The biological scaffold becomes entrapped as the inorganic material forms, prompting an investigation into biosilicification as a mechanism for enzyme immobilization. Initial studies focused on the immobilization of enzymes within silica nanoparticles that were formed by reaction of a silicate precursor with a short silica-forming peptide (R5) [73]. The peptide is a synthetic derivative of a naturally occurring silaffin protein, found in the silica skeleton of the marine diatom *Cylindrotheca fusiformis* [69,71,74,75]. The reaction forms a network of fused silica nanospheres with a diameter of approximately 500 nm. The silica particles form rapidly and entrap the scaffold peptide and any other material that is contained within the reaction mixture. Preliminary experiments, for example, showed the successful encapsulation of a range of enzymes and also non-biological components such as; magnetic cobalt platinum, CdSe/ZnS nanoparticles (quantum dots) and iron oxide nanoparticles [60,73]. The resulting composites retained their fluorescence properties (for quantum dots) and magnetic properties (for iron oxide) respectively (Figure 2).

Figure 2. Formation of silica particles using a synthetic peptide

Silicate precursor in phosphate buffer (A) reacts with the R5 peptide to form silica (B), which can be pelleted by centrifugation (C) to reveal a matrix of silica particles as observed by SEM (D). The silica particles can entrap additional molecules during the reaction, e.g. quantum dots (E) or magnetic nanoparticles (F). *Image (E) and (F) reproduced with permission from the Royal Society of Chemistry 2004, (http://dx.doi.org/10.1039/b404586f) [60]*



Despite the versatility of the synthetic peptide (R5), subsequent studies demonstrated that silica-formation could also be catalyzed by a wide range of cationic amine-rich molecules including silica-binding peptides, polymers such as polyethyleneimine and poly-L-lysine (PLL), cysteamine and proteins such as silicatein and lysozyme (Table II). A similar study, for example, reported the encapsulation of firefly luciferase and green and blue fluorescent proteins in silica particles formed using cysteamine as the scaffold catalyst [76].

Table II. Characteristics of silica formation from various cationic species

Cationic mediator	Silica Characteristics	Reference
Silaffin and R5 peptide	Spherical particles 500-700 nm	[69,71]
Lysozyme	Spherical particles ~500 nm	[76,77]
Silica-binding peptides	Spherical particles 250-500 nm	[78]
Silicateins	Silica sheets along protein filaments	[67,68]
Block co-polypeptides	Various morphologies from spheres to columns depending upon the precursor	[79]
Poly-L-lysine	Various morphologies including nanoparticles (50-100 nm) and hexagonal platelets (0.5 -1 μm)	[80-85]
Polyethyleneimine	Various morphologies including spheres, ribbons, nanofibres and platelets	[86-89]
Amine-terminated dendrimers	Spherical particles, size dependent upon reaction conditions	[90]
Spermine/Spermidine	Spherical particles 500 nm to 1μm	[91]
Cysteamine	Spherical particles ~40-100 nm	[92]

In all cases, the silica forms at ambient environmental conditions, but with a range of morphologies dependent upon the nature of the precursor and the reaction conditions. The mild reaction conditions are compatible with enzyme immobilization and allow retention of high levels of enzyme activity. The encapsulation of biomolecules within silica nanoparticles using this technique has now been investigated extensively and demonstrated for a wide variety of enzymes (Table III) [60,73,88,93-98].

The primary advantage of the biological synthesis of silica is the benign reaction conditions (ambient temperatures, physiological pH range and aqueous solvents) which favor retention of biological activity. The mechanism of silica formation and simultaneous biological templating within these systems still requires further elucidation as it is presently unclear whether the majority of the enzyme is encapsulated within the silica matrix or simply adsorbed to the silica structure once it is formed. This is an important distinction when one considers the question of enzyme activity particularly in respect to diffusion and mass transfer limitations, which will differ greatly between i) enzyme molecules that form an integral part of the support matrix and ii) enzyme molecules that are adsorbed to the surface by electrostatic or hydrophobic/hydrophilic interactions. The silicification reaction, however, imparts remarkable stability to the encapsulated enzyme in terms of both operational stability (reuse during catalysis) and inherent catalytic stability (resistance to denaturation).

Table III. Immobilization efficiency of a range of enzymes in silica nanoparticles

***Efficiency is cited as retention of enzyme activity,

† Immobilization efficiency dependent upon reaction conditions

Enzyme	Silica mediator	Immobilization efficiency*	Reference
Butyrylcholinesterase	R5, R5-His_6 Lysozyme	>90%	[73,76,97]
Catalase	R5	100%	[60]
Horseradish peroxidase	R5	>90%	[60]
Soybean peroxidase	R5	65 - 85%	[98]
Hydroxylamino-benzene mutase	R5	44 - 67%	[95,98]
Nitrobenzene nitroreductase	PEI	~60% - 80%	[88,94]
Organophosphate Hydrolase	Lysozyme	ND	[96]
Glucose-6- dehydrogenase	PEI	~33%	[94]
β-galactosidase	R5	~90%	[99]
Lipase	PEI	<10 %	[89]
Glucose Isomerase	PEI	>40 %	[89]
Glucose Oxidase	Dendrimers	4 - 54%†	[90]
Horseradish peroxidase	Dendrimers	13 - 40%†	[90]
Luciferase	Cysteamine	87%	[92]
Luciferase	Ethanolamine	17%	[92]
GFP	Ethanolamine	ND	[92]

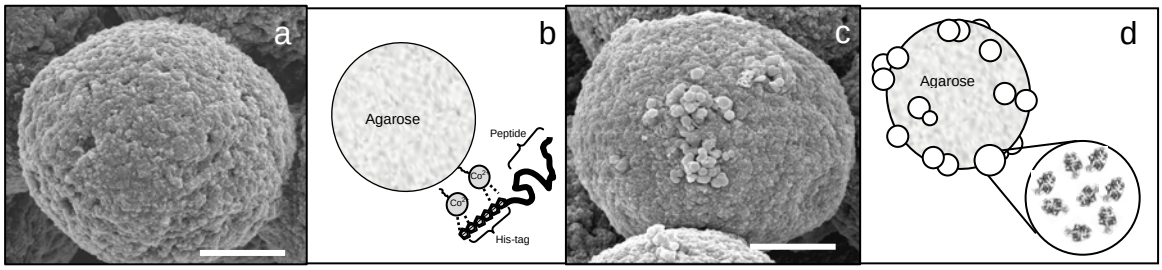
Butyrylcholinesterase (BuChE), for example, shows superior thermal stability when encapsulated in silica nanospheres. The thermostability of the immobilized enzyme was greatly enhanced resulting in a resistance to denaturation at temperatures up to 65^{o}C; conditions which caused rapid denaturation of the native enzyme [73]. The stability is attributed to the confinement of the enzyme within the silica matrix, which theoretically prevents the protein from unfolding. Initial studies focused on the immobilization of BuChE due to the potential applications of this enzyme in the development of biosensors. The immobilization of BuChE in silica proved extremely versatile and suited to continuous-flow systems; for screening drug potency and for development of organophosphate detection systems [97]. The use of silica encapsulation for continuous flow-systems will be discussed in more detail below.

Continuous flow systems for silica-immobilized enzymes

Immobilized enzymes that are integrated into flow-through systems are typically termed Immobilized Enzyme Reactors (IMERs) and numerous examples of IMERs with particular relevance to biocatalysis have now been reported [88,100-115]. The integration of immobilized enzymes to continuous flow-systems is an important consideration in biocatalyst design to enable reuse and continuous recycling. Current IMER configurations however, often exhibit specific drawbacks such as low loading capacity and long preparation times [116-119]. IMERs consisting of immobilized cholinesterase enzymes have been investigated in drug screening to identify inhibitors for treatment of neural disorders, such as Alzheimer's disease. Acetyl-cholinesterase (AChE), for example, catalyses the hydrolysis of acetylcholine; a neurotransmitter in the central

nervous system and inhibition of the enzyme provides a mechanism for treatment of neurodegenerative diseases [118]. Bartolini *et al*., recently reported a comparison of silica-packed columns versus monolithic columns for IMER preparation with immobilized AChE [120]. The resulting monolith columns showed good stability but the preparation time was slow and loading capacities were low (~3%). The silica-packed column provided a greater immobilization yield (~29%) in a shorter preparation time and with a significant reduction in cost. By comparison, immobilization of BuChE in silica nanoparticles using the biosilicification method was complete in less than 1 hour with an immobilization efficiency of 100% and a high loading capacity for enzyme [97]. The IMER was formed by binding the silica particles (containing immobilized enzyme) to a commercially available pre-packed metal affinity column. The column contains large agarose beads designed for optimal flow conditions that are charged with cobalt metal ions. A homologue of the silica-forming (R5) peptide is synthesized with a 'tail' of six histidine residues that selectively bind to the cobalt coated agarose beads. Silica formation occurs at the site of the bound peptide, resulting in formation of silica nanospheres attached directly within the column. Exogenous enzyme added during the reaction becomes entrained as the silica particles form and simultaneously attach to the surface of the agarose beads (Figure 3).

Figure 3. Formation of biosilica *in situ* to form an immobilized enzyme reactor
Agarose beads (a) coated in cobalt, bind a silica-forming peptide (b). Silica nanospheres form at the surface (c). Additional enzyme added during the reaction becomes entrapped within the silica (d). Scale bar = 3.75 μm (a,c). Adapted from [97] and [121]



IMER columns developed using this method were stable for over 2 days of continuous use and amenable to a wide range of flow rates up to 3 ml/min. The reusability is a primary advantage as it significantly reduces the amount of enzyme required for analysis, providing an opportunity to conduct biocatalyst with expensive biomolecules which would be cost-prohibitive without extensive recycle. In addition, the histidine-tag is placed on the silica-forming peptide rather than on the protein of interest, eliminating any need for recombinant modification of the biocatalyst in order to utilize this technique [97]. The biosilica-IMER system was demonstrated for immobilized BuChE as a technique for screening the drug potency of a range of cholinesterase inhibitors. Cholinesterase enzymes find primary application in biosensor development and drug screening but applications in biocatalysis have yet to be defined. BuChE, for example, shows remarkable substrate specificity and can detoxify a diverse range of drugs including cocaine and heroin. As noted by Khosla and Harbury, such substrate specificity may provide a very versatile catalyst for enzymatic synthesis of novel molecules [122].

Cholinesterase is just one example of the many enzymes that exhibit specificity for their natural substrates as well as a wide variety of substrate homologues that makes them equally applicable to detection systems as well as biocatalysis. In fact, detection and biocatalysis often offer parallel applications; the formation of reaction intermediates is often the basis for biological detection and capturing the same reaction intermediates (or near homologues) is the inherent goal of biocatalysis. The use of nitroreductase enzymes, for example, has been demonstrated for utility in biocatalysis and drug screening by encapsulating the enzyme within silica nanospheres.

Nitroreductase for drug screening

The use of nitroreductase enzymes for directed enzyme-prodrug therapy (DEPT) consists of directing an exogenous nitroreductase to specific cells; where it activates a prodrug to a cytotoxic derivative [123]. Despite the potential pharmacological role of nitroreductases, there are few literature reports demonstrating the immobilization of such enzymes. The majority of the work focuses on biosensing applications [124]. A continuous flow system containing immobilized nitroreductase not only would potentially allow for the examination of different prodrug candidates but also permit the study of selectivity, specificity and kinetic parameters of putative DEPT enzymes with minimal amounts of often expensive substrates. As such, the nitrobenzene nitroreductase from the bacterial isolate *Pseudomonas pseudoalcaligenes* JS45 was encapsulated in silica nanospheres using the silicification method described above. The immobilized enzyme was packed into a microfluidic column and used to determine the pharmacological activity of nitroreductase for activation of cancer prodrugs and pro-antibiotics [62]. The immobilization system involved the use of PEI as the amine-rich catalyst used to drive the formation of the silica particles [62]. SEM analysis of the derivative revealed the formation of a matrix of interconnected silica particles of approximately 0.5 -1.0 μm diameter. The immobilized nitroreductase expressed 60% of the entrapped activity and the kinetic parameters of the enzyme were not affected during immobilization. Moreover, the preparation was thermo-stable and provided a fortuitous enhancement in solvent stability of the immobilized protein. Once characterized, the silica entrapped nitroreductase was packed in a stainless steel microcolumn (20 x 2 mm) and tested for the continuous conversion of 5-azirinyl-2,4-dinitrobenzamide (CB1954), a cancer prodrug [123,125], a proantibiotic; nitrofurazone[126,127] and the enzyme's natural substrate; nitrobenzene. At low flow rates (1 μL/min), the three substrates were converted stoichiometrically. In addition, the conversion of nitrobenzene was maintained continuously (>90%) for more than 3 days at room temperature, at a flow rate of 5 μL/min.

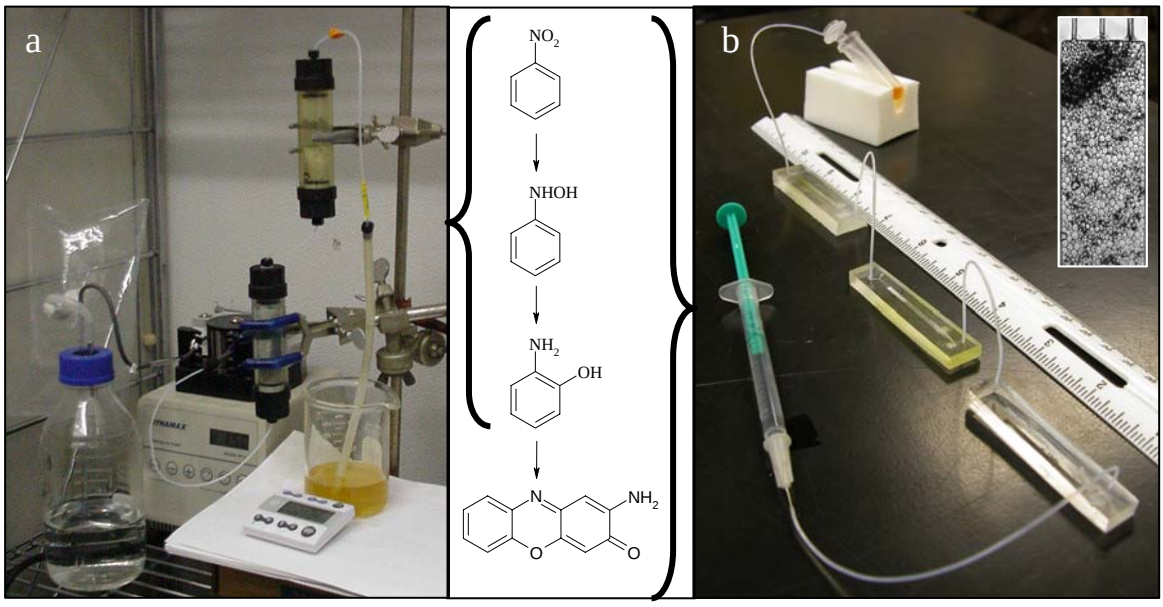
Nitroreductase for biocatalysis

In addition to prodrug activation, the nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45 also catalyzes the oxidation of nitroarenes. A suite of enzymes systematically convert the nitro-moiety to intermediate metabolites. In the first step, the nitro-moiety of nitrobenzene (the native substrate), for example, is reduced to hydroxylaminobenzene (HAB) via the nitrobenzene nitroreductase. The resulting HAB intermediate is further metabolized to aminophenol via a novel intermolecular rearrangement reaction, catalyzed by HAB-mutase. Both enzymes have proven to be

versatile catalysts for biocatalysis and have been demonstrated for the synthesis of a range of substituted aminophenols from nitroarenes [95,128-131]. Both nitrobenzene nitroreductase and HAB-mutase can be encapsulated in biosilica and integrated into systems that can operate at flow rates ranging from a few microlitres per hour up to milliliters per minute depending upon the application [88,95,97,98].

Nitrobenzene nitroreductase can be replaced by metallic zinc to catalyze the reduction of nitrobenzene [132]. In an initial study, we demonstrated that a column (10 ml volume) containing zinc could be connected in series to a second column containing HAB-mutase immobilized in biosilica (Figure 4a). Flow rates of up to 0.5 ml/min resulted in good conversion of nitrobenzene to aminophenol. An alternate microfluidic system was also demonstrated, operating at a much smaller scale with reaction volumes of only a few microlitres. Microfluidic systems were devised by packing the silica particles within a PDMS based chip containing a three channel weir; specifically designed to retain the silica encapsulated catalysts within the flow channel (Figure 4b).

Figure 4. Biocatalysis of nitrobenzene in large and small scale systems
Zinc and silica-encapsulated HAB-mutase in a sequential flow through system for synthesis of aminophenol from nitrobenzene (a), and same in a microfluidic system with silica-encapsulated soybean peroxidase as an additional step to synthesize aminophenoxazinone. Inset to panel b shows the weir integrated in chips to retain silica particles.



Simple packaging of this kind has the advantage that there is no requirement for surface modifications to allow enzyme attachment, greatly reducing preparation time and enhancing the loading capacity of the reaction system [133]. A chip containing zinc (to catalyze the reduction of nitrobenzene to HAB) was connected in series to a second chip containing HAB-mutase, resulting in the formation of aminophenol from nitrobenzene as before. In the microfluidic system, however, an additional enzyme was immobilized in silica and entrained within a third chip. The third chip contains silica-encapsulated soybean peroxidase that catalyses the polymerization of *ortho*-aminophenol (product of the second chip) to form 2-aminophenoxazin-3-one [134]. The final product is an

intermediate in the synthesis of actinomycins; a potentially interesting group of antibiotics with anti-fungal and anti-tumor properties [135-137]. The two enzyme-based steps in sequence resulted in the conversion of HAB to APO with good conversion efficiency (~52%) at modest flow rates (100 – 150 μl/h). The three step chemoenzymatic microfluidic platform with a zinc chip was also demonstrated, but conversion efficiencies were limited by the ability to balance complete reaction conversion with a flow rate that was suited to all three components. The zinc chip, for example, catalyzed optimal conversions at high flow rates that did not favor complete conversion in the subsequent reaction steps. As such, a low conversion efficiency of 25% was reported (although not optimal). Reducing the flow rate too much resulted in the formation of aniline as an unwanted byproduct in preference to HAB. The PDMS chips were also susceptible to some adsorption and losses due to the volatility of the substrate. Despite the low overall yields of the chemoenzymatic steps in series, the system provided a potential method for screening the conversion of nitroarenes into corresponding phenoxazinone products. In addition, single unit reactors containing enzyme or metal catalysts can be mixed and matched to create a variety of synthetic pathways for rapid synthesis and screening.

Future Directions

The majority of enzyme immobilization studies using this methodology have been restricted to the encapsulation of enzymes within silica matrices. The biological synthesis of inorganic oxides, however, is not restricted to silica. Lysozyme, for example, can also template the formation of titanium dioxide; the structure and morphology of which can be varied depending upon the nature of the precursor [76]. Lysozyme as a scaffold for silica formation provides an interesting functionality, as lysozyme exhibits antimicrobial activity. Lysozyme directs the formation of both silica and titania nanoparticles, simultaneously entrapping itself in an active form and retaining its antimicrobial activity [76]. Lysozyme/silica composites therefore provide antifouling properties to the encapsulated catalysts, for potential use as antibacterial coatings. The morphology of the inorganic matrix can also be varied to create more functionalized and three-dimensional structures [138]. Advantages provided by changes in morphology, for example, could be linked to specific attachment methods and increased surface area for catalysis. In addition, the use of different precursors to drive the formation of the silica particle may also provide a protective microenvironment which could increase the stability of the immobilized enzymes towards organic solvents as shown in Berne *et al*. [62]. Additionally, silica spheres may act as “smart” supports that change their solubility properties in response to an environmental stimulus, e.g. silica particles may be dissolved in alkaline conditions releasing a soluble enzyme and increasing their scope of applications [139].

Apart from the above mentioned advantages of the silica encapsulation of enzymes (improvements in biocatalyst stability, good physical properties for flow through applications etc) this system also provides an excellent method for the co-immobilization of enzymes. The essence of the system is the co-immobilization of generally two proteins; one to catalyze the formation of silica and a second that is the enzyme to be immobilized. The system therefore provides an ideal opportunity to immobilize a number of enzymes to function in tandem. The fact that any enzyme added

during the biosilification reaction becomes entrapped, with often high loading capacities, allows for close confinement of sequentially acting enzymes which may increase the catalytic efficiency of conversions due to a dramatic reduction in the diffusion time of the substrate. This concept was demonstrated by Betancor *et al.*, who reported the use of silica nanoparticles to encapsulate nitrobenzene nitroreductase and glucose-6-phosphate dehydrogenase simultaneously to produce a system that recycles NADPH *in situ* to maintain the continuous reduction of nitrobenzene to hydroxylaminobenzene (HAB) [140]. NADPH was supplied to initiate the reaction and coupling of NBNR and G6PDH activities was evidenced by the continuous formation of HAB for 8 hours without further addition of cofactor. This technique could potentially be extended to multi-enzyme composites to mimic metabolic pathways or to generate encapsulated protein microarrays [134,141-144]. Based on all the advantages demonstrated, the main potential of this immobilization methodology will likely be the versatility to a wide range of biomolecules, particularly with respect to the co-immobilization of a variety of potentially interchangeable multi-enzyme configurations for preparation of robust biocatalysts or biosensing composites.

CONCLUSION

Enzyme immobilization methods have been widely investigated for many years, but recent developments in stabilizing enzymes within biologically templated inorganic matrices substantially extends the range of operational stabilities. Nano-sized materials offer a number of intrinsic advantages such as high surface areas which lead to high loading capacities. Silica-encapsulation has proven to be a versatile method for immobilizing biocatalytic activity and is applicable to a wide range of biomolecules. We anticipate that the resulting silica-encapsulated catalysts will find significant and widespread application in the design of biosensors and for biocatalysis and drug discovery. Immobilization in silica has so far been restricted to enzymes but the feasibility of the approach for the encapsulation and stabilization of other biomolecules requires further study. For example, biomolecules such as antibodies, phage, DNA and the encapsulation of multi-enzyme systems all have potential applications as immunoassays, arrays and whole cell mimics.

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